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ARL-RP-0502 **August 2014**

A reprint from *Analytical Chemistry, Vol. 7, No. 10, 9452–9460, 2013.*

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14. ABSTRACT
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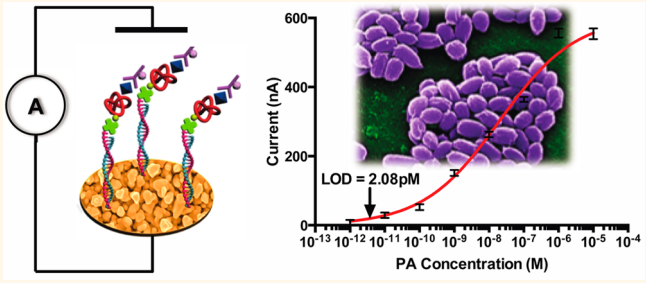
ABSTRACT We report on a robust and sensitive approach for detecting protective antigen (PA) exotoxin from *Bacillus anthracis* in complex media. A peptide-based capture agent against PA was developed by improving a bacteria display-developed peptide into a highly selective biligand through *in situ* click screening against a large, chemically synthesized peptide library. This biligand was coupled with an electrochemical enzyme-linked immunosorbent assay utilizing nanostructured gold electrodes. The resultant assay yielded a limit of detection of PA of 170 pg/mL (2.1 pM) in buffer, with minimal sensitivity reduction in 1% serum. The powdered capture agent could be stably stored for several days at 65 °C, and the full electrochemical biosensor showed no loss of performance after extended storage at 40 °C. The engineered stability and specificity of this assay should be extendable to other cases in which biomolecular detection in demanding environments is required.



KEYWORDS: pathogens · biosensing · electrochemistry · nanomaterial · ELISA · anthrax · protein capture agents

There exists an unmet need for rapid, sensitive, and field stable assays for pathogen detection. *Bacillus anthracis* is the causative agent of anthrax poisoning. This Gram-positive bacterium secretes a tripartite toxin including a cell-binding protective antigen (PA), and the delivered toxins edema factor (EF) and lethal factor (LF).[1] Anthrax poisoning has high mortality and, when delivered in the form of *B. anthracis* spores, has a very high environmental stability. These attributes make the detection of anthrax a priority with regards to issues ranging from food safety to bioterrorism. Current detection methods in use include detection of the *B. anthracis* spore biomarker calcium dipicolinate (DPA),[2] (antibody-based) immunoassays for detecting PA and LF,[3,4] and amplification and sequencing of characteristic nucleic acids.[5]

PA is an important target for detection, and so it is not surprising that various biosensor platforms for the detection of PA have been reported, with peptide binders (developed using bacterial or phage display techniques) coupled to graphene-, carbon nanotube-, and ZnO-based electrodes or surface enhanced Raman spectrometer (SERS) substrates achieving limits of detection under 20 pg/mL.[6−9] These systems are, by and large, not appropriate for field use, due to their experimental complexity and/or unproven capacity to detect PA out of complex media such as blood serum. Other immunoassay techniques have been reported which use various enzyme-linked immunosorbent assay (ELISA) amplification and sensitivity-boosting methods, enabling Europium-conjugated and AlphaLISA antibody-based immunoassays to increase sensitivity to as high as 100−200 pg/mL out of dilute serum.[3,10] These systems have demonstrated detection and quantification of PA out of clinically

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relevant serum samples, but their use of antibodies for the capture and detection of PA can limit their performance under physical conditions far removed from the laboratory. In fact, no PA assay that exhibits high performance under physically demanding conditions has been reported.

Of the five fatalities in the 2001 bioterrorism attacks against the United States involving anthrax, the causative agent was only identified in one individual before death.[11] This points to the need for high performance assays for the analysis of complex samples under such demanding physical conditions. We report on the development of a stable, synthetic peptide-based capture agent against PA that, when coupled with a specially designed electrochemical assay, permits the detection of PA with a limit of detection (LoD) of 170 pg/mL (2.1 pM), with little sensitivity loss in diluted human serum. The powdered capture agent may be safely stored in a sealed environment at up to 65 °C for several days.

The gold standard reagents for protein detection are monoclonal antibodies (mAbs). Although mAbs can exhibit impressive affinity and specificity, as biological reagents, they can also be plagued by high cost, batch-to-batch variability, and poor stability.[12,13] Alternative capture agent technologies, such as phage or bacterial display peptides and aptamers,[14,15] address some of these problems, although achieving high target selectivity is often challenging. We have recently developed the technique of iterative *in situ* click chemistry for the production of what we call protein catalyzed capture agents, or PCC Agents.[16]

The technique uses the protein target itself as a highly selective catalytic scaffold for promoting the Huisgen 1,3-dipolar cycloaddition (click) reaction between two substrates (one presents an azide, the other an acetylene group) to form a covalent triazole linkage.[17] We report on a variation of earlier approaches[16,18−19] by using, as one of the substrates, a peptide that was identified *via* bacterial display screening techniques. That peptide was chemically altered to present an acetylene group, plus a biotin handle linked through a polyethylene glycol oligomer. These modifications make it an anchor ligand for an *in situ* click screen[20] (Figure 1a). Such a screen was then executed to identify a second ligand. For that screen, the anchor, the target protein, and a large one-bead-one compound (OBOC) library[21] of azide presenting D-stereoisomer peptides were co-incubated. A hit bead is defined as one that contains a candidate second ligand coupled, *via* a triazole, to the anchor. Thus, the bead library is analyzed, using the biotin handle on the anchor ligand, to identify those beads that contain this product[18] as well as to remove those potential hits that exhibit nonselective binding.[16,18−20] Hits are sequenced to identify candidate second ligands. Biligands are prepared from those candidates by clicking together the initial first ligand and candidate second ligands, and then tested for affinity and selectivity to identify a consensus PCC Agent. The process can be iterated to develop triligands, *etc.*, but for this work, the biligand PCC Agent exhibited sufficient performance metrics for the end-use assay. The final PCC Agent retains the biotin handle, which allows for incorporation into standard immunoassay formats. The electrochemical detection method we utilize is conceptually similar to one such standard format.

We explored an electrochemical variant[22−24] of sandwich-type Enzyme-linked Immunosorbent Assay (ELISA). Standard (optical) ELISAs use an enzyme label which catalyzes the turnover of a colored substrate. Electrochemical ELISAs require an electrochemically active product. We use *p*-aminophenyl phosphate (APP), which, as a substrate for the enzyme label alkaline phosphatase (ALP),[25] produces the electrochemically active product, *p*-aminophenol. This product is detected at low redox potentials that are well separated from those of its parent substrate.

We employed a gold-black nanostructured substrate as the working electrode to significantly increase the available electrode surface area,[26] and thus amplify the electrochemical signal used for PA detection. A drawback of electrochemical ELISAs is that the layers of immobilization and recognition species that cover the electrode surface can inhibit diffusion-limited electron transfer from the redox-active substrate to the electrode.[22] We used the DNA-encoded antibody library technique[27,28] to prepare a dense DNA scaffold for a high-density display of the PCC Agent.[27] An advantage of this scaffold is that electron transfer to the redox-active reporter substrate is facilitated by the DNA-duplex monolayer.[29] The detection limit of the electrochemical assay was sharply decreased (to ∼2 pM) relative to an otherwise equivalent optical ELISA assay (∼10 nM). Electrochemistry also provided a straightforward approach toward detecting PA in optically dense or turgid samples, such as 5% human serum, and exhibited excellent physical stability.

RESULTS AND DISCUSSION

Anti-PA Capture Agent Development and Characterization. The *in situ click* screen to identify an anti-PA PCC Agent biligand started with an initial, or first ligand that was selected from a group of bacterial display 15-mer peptides. Those peptides were generated by screening an *Escherichia coli* bacterial display library against PA.[30] Each peptide was expressed on the surface of *E. coli* and qualitatively tested against both PA and off-target proteins (Supporting Experimental Methods). The peptide with the best combination of high PA-binding and low off-target interactions was selected for modification into an anchor ligand. To this end, the peptide was synthesized with a C-terminal biotin label, and an

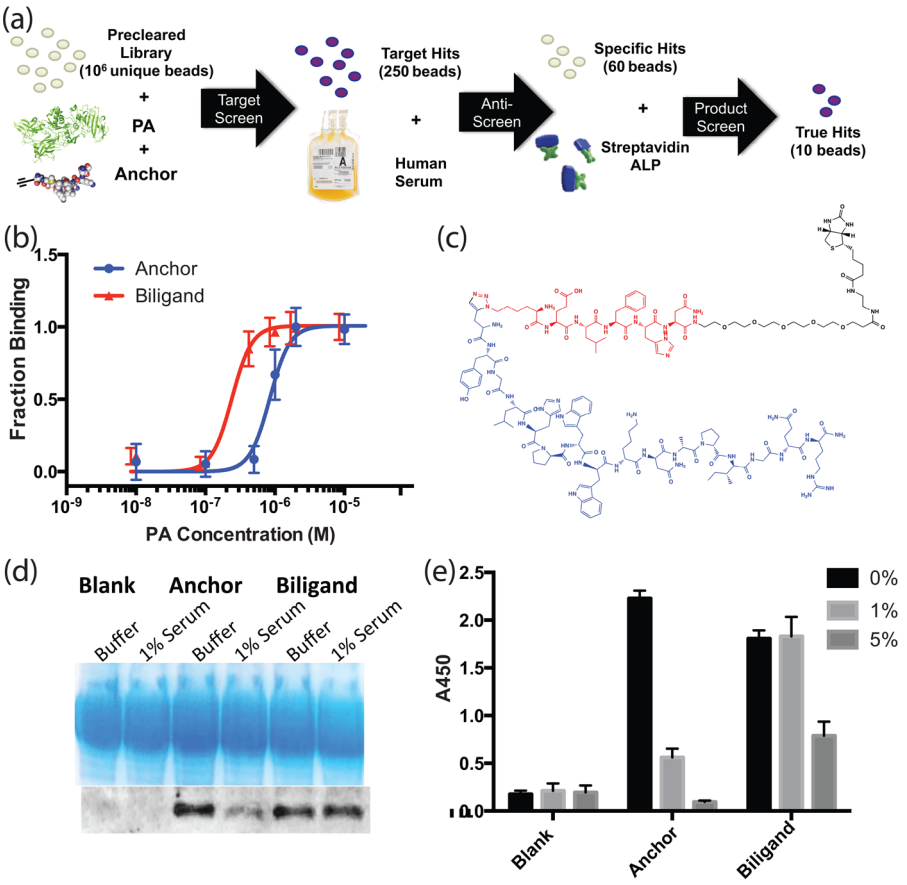

Figure 1. Development and characterization of an anti-*Bacillus anthracis* Protective Antigen biligand PCC Agent. (a) The scheme for the *in situ* click screen used to develop the biligand. The target screen identifies as hits those beads to which PA binds. The anti-screen (carried out in 1% human serum) removes those hits that also bind to serum proteins. The product screen further reduces the number of hits by detecting only those beads that contain the *in situ* click product. (b) Colorimetric ELISA binding isotherm showing differential binding between the anchor peptide and the PCC Agent biligand. (c) Consensus structure of the biligand PCC Agent. (d) Western blotting (immunoprecipitation) selectivity assay exhibiting differential pull-down of PA from buffer or 1% serum by the anchor and biligand capture agents (plus a blank). (e) Single-point competitive ELISA in increasing concentrations of human serum showing qualitatively improved performance of the biligand relative to the anchor.

N-terminal propargyl glycine to form Anchor-1 (Figure S1). The affinity and selectivity of Anchor-1 for PA was evaluated *via* immunoprecipitation and colorimetric ELISA assays (Figure 1b,d,e).

The *in situ* click screens used to develop the anti-PA biligand PCC agent are summarized in Figure 1a. The preclear step removes those ~10% of the beads which bind to non-PA reagents used in the screening process. This is followed by a target screen that yielded ~250 hit beads (0.02%) which exhibited binding to PA. That number of hits was reduced by about 75% by screening for those hit beads that exhibited binding to any off-target proteins in 1% human serum. Finally, each of the remaining 60 hit beads was screened for the presence of on-bead clicked product.[18] That product screen yielded 8 hits, each of which was sequenced by Edman degradation (Table S1). These second ligands exhibited a high homology, which is a signature of a successful *in situ* click screen. Candidate PCC Agent biligands were prepared using the Cu(I) catalyzed reaction to click the anchor peptide with each of the second ligands, and each of the resultant 8 biligands was tested in binding assays for selectivity and affinity to PA. Three biligands exhibited significantly improved selectivity in an immunoprecipitation assay for the detection of PA spiked in 1% serum (Figure 1d and Figure S2). The affinities of those three biligands were estimated, relative to Anchor-1, using single-component ELISA assays (Supporting Information Methods). The consensus biligand (Figure 1c) exhibited a 3−4-fold affinity improvement (capture IC_{50} = 190 nM) (Figure 1b). The advantage of the *in situ* click screen for improving the original peptide ligand is implied in the hit frequencies through the various screening steps. Note that only 1 in 25 target hits actual yields a true hit. Target hits are often simply 'sticky', or nonselective peptides. Even the anti-screen step only reduced the numbers of target hits by a factor of 4. The final product screen is, in many ways, a selectivity screen, since it only selects those peptides that can arrange themselves on the protein surface in just the right manner to promote the click reaction.

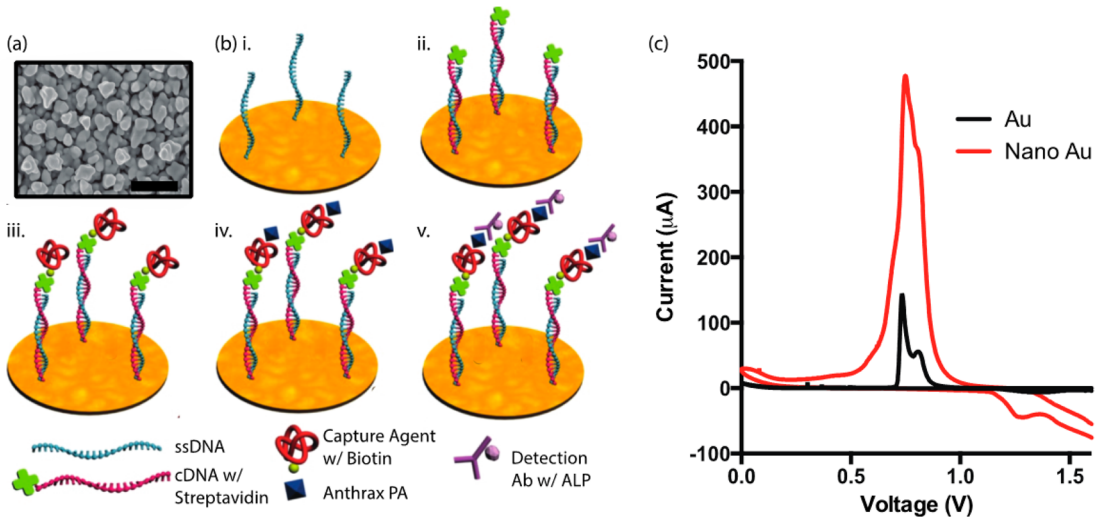

Figure 2. Preparation and characterization of the electrochemical ELISA platform. (a) SEM micrograph captured using a Hitachi S4700 of the nanostructured gold electrode, scale bar is 1 μm. (b) Surface chemistry preparation scheme to prepare the nanostructured electrodes for the ELISA assay: (i) thiolated ssDNA monolayer formation; (ii) hybridization of streptavidin-modified cDNA; (iii) immobilization of biotin-modified PCC Agent; (iv) capture of PA with PCC Agent; (v) detection of captured PA with ALP-linked antibody. (c) Current−voltage plot of bare (black line) and nanostructured (red line) gold electrode.

We quantitated the specificity of the consensus PCC Agent relative to Anchor-1 using two selectivity assays. For the first, we measured the off-target binding of the two capture agents to other secreted pathogenic proteins, relative to the response to PA. The second selectivity assay was a competitive (colorimetric) ELISA in which the detection of PA was challenged by the presence of human serum (Figure 1d). In both assays, the PCC Agent showed significantly superior performance. Anchor-1 exhibited off-target binding to the secreted Lethal Factor from *B. anthracis*, and the capsid protein L1r from *Vaccinia* virus (Figure S3), and showed little capacity to capture PA from a 1% serum background. By contrast, the PCC Agent exhibited very little cross-reactivity with the other pathogenic proteins, and captured PA in even up to 5% human serum. These observations suggest that the second ligand branch identified through the *in situ* screen contributes destabilizing interactions to off-target proteins, and modest stabilizing interactions to PA. Previous studies have found consistent results when designing highly specific ligands using negative design, including examples of protein−protein interactions, and peptide multiligand interactions.[18,31]

Development and Characterization of Electrochemical Platform. A nanostructured gold-black electrode was fabricated through electroplating of gold salts on to a bare gold electrode. The deposition conditions were optimized by measuring the active surface area by cyclic voltammetry and noncontact AFM upon varying deposition time and voltages (Figure 2c, Figure S4).[32,33] For comparison, the bare gold electrode and the nanostructured gold electrode were electrochemically characterized in 0.5 M H_2SO_4 by integrating the voltammogram associated with gold oxide reduction. Assuming a specific charge of 388 μC/cm^2 is required for gold oxide reduction,[34] the optimal nanostructured gold electrode had a total active surface area of 122.47 $\pm$ 1.5 mm^2 after 400 s of deposition, while the unmodified electrode had a total active surface area of 20.4 $\pm$ 0.8 mm^2. The 6-fold signal enhancement reflects the enhanced surface area of the nanostructured gold electrode (Figure 2c; Figures S4 and S5). The electrode was further imaged by noncontact AFM and SEM, which showed a roughened, porous surface with nanostructures on the scale of 50−100 nm (Figure 2a).

Each step in preparing the functionalized electrode for electrochemical detection of PA was optimized, including thiol-modified DNA monolayer deposition, cDNA hybridization, capture agent immobilization, PA-incubation time, ALP-conjugated detection antibody incubation, and electrochemical development. Forming a high quality DNA monolayer helps ensure a high-density presentation of the biligand capture agent, which directly influences assay performance. The conditions for the formation of the DNA duplex were optimized, using fluorescence microscopy of Cy3-labeled DNA as a metric (Figure S6). This approach was based upon literature protocols[35] in which such fluorescence measurements were correlated with near angle X-ray absorption fine structure (NEXAFS) characterizations of a DNA duplex monolayer.[36] For optimization of the other parameters, PA incubation and detection with an ALP-labeled antibody were performed and conditions were chosen which maximized the peak centered at 0.065 V *vs* Ag/AgCl corresponding to the oxidation of APP. This data can be found in Figures S6−S9 in the Supporting Information.

Assay Performance. We characterized the electrochemical ELISA using titrations of PA at concentrations ranging between 1 pM and 10 μM. The current was

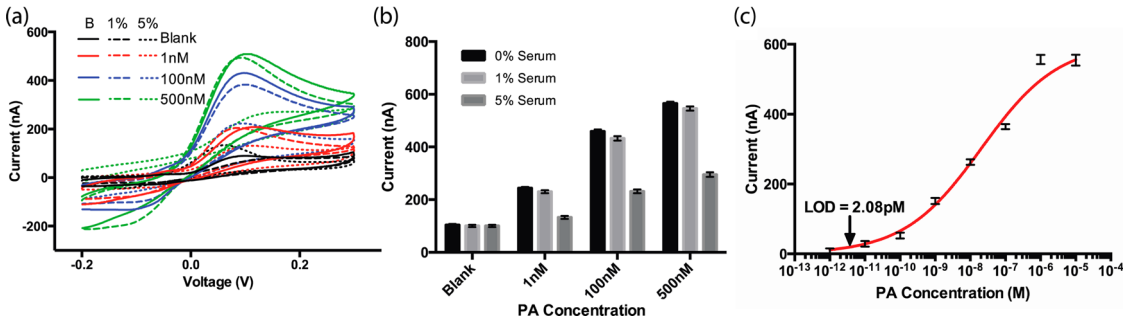

Figure 3. Performance of the electrochemical ELISA assay for PA detection. (a) Current−voltage curves used for the detection of PA from diluted serum samples: B, buffer; 1%, 1% serum; 5%, 5% serum. (b) Peak currents generated using to report the binding of PA, as carried out in various indicated concentrations of human serum. (c) Binding isotherm of PA measured in buffer solution using the electrochemical ELISA. The average background was subtracted to facilitate fitting to the Hill function.

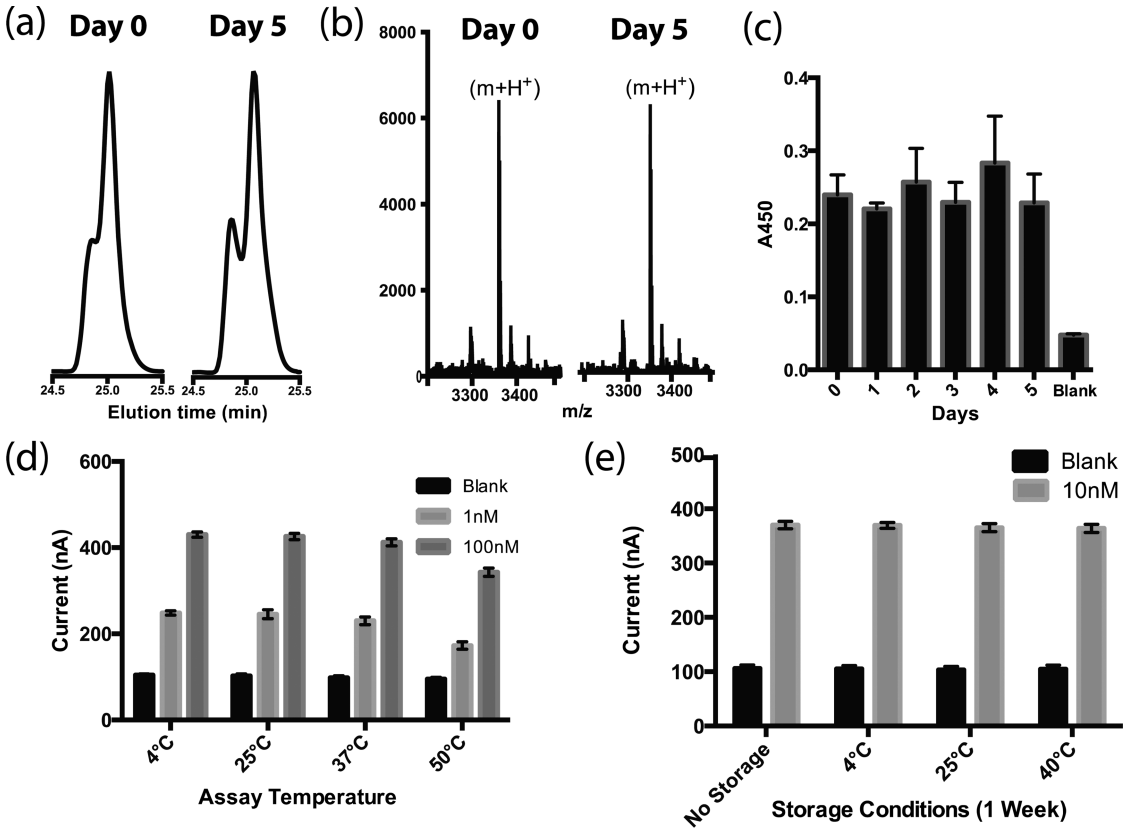

Figure 4. Thermal stability of the PCC Agent and the electrochemical ELISA platform. Analytical HPLC (a) and MALDI-TOF (b) traces of biligand capture agent with no thermal treatment, and after 5 days of storage as a powder at 65 °C under nitrogen. (c) Single-point ELISA assays, performed with the PCC Agent after storage as a powder for 0−5 days at 65 °C under nitrogen, reveal no evidence of performance degradation during storage. (d) Electrochemical ELISA assay performed at varying temperatures, indicating the robustness of the integrated assay platform. The slight drop-off in performance at 50 °C likely originates from the enzymatic detection. (e) Electrochemical ELISA signals measured at room temperature after storage of the fully assembled nanostructured electrode at varying temperatures for one week.

monitored at the APP peak centered at 0.065 V *vs* Ag/AgCl. Calibration plots (in buffer) were highly reproducible and were fit to a Hill function (Hill coefficient = 0.39; IC_{50} = 80 nM). This low Hill coefficient implies a longer range of quantification for the electrochemical ELISA relative to the optical ELISA (Hill coefficient = 2) and is similar to other standard curves collected from electrochemical ELISA platforms.[22] The range of quantification can be seen in Figure 3c extending from approximately 100 pM to 1 μM with a sensitivity of ~100 nA/decade over at least 3 orders of magnitude. The LoD, as determined by when the signal level is 3× the standard experimental error, was 2.1 ± 1.5 pM (170 pg/mL). This low detection limit is possible because oxidation currents at a PA concentration of zero were low (5 nA or less) and highly reproducible.

We then moved toward characterizing the electrochemical ELISA in the environment of diluted human

serum spiked with PA toxin. Human serum contains approximately 50−85 g/L of protein and, so, constitutes a demanding environment for the assay. The assay performed well at up to 5% human serum, showing a similar sensitivity decrease as was observed in the conventional ELISA. Even with this decrease, the LoD was calculated to be 2.2 pM from 1% serum, and 181 pM from 5% serum. These LoD values were estimated by assuming that the Hill function coefficients from the fit of the spiked buffer solution isotherm were unchanged in the 1% or 5% serum isotherms, but the IC_{50} value was allowed to change. Since the concentration of biomolecular interferents is approximately 200 μM in 5% serum, this demonstrates at least a million-fold sensitivity preference for PA. Current evidence, based on animal studies, suggests that PA concentrations reach approximately 0.1 μg/mL (1.2 nM) in the blood of infected individuals during the acute stage of disease development.[9] In 1% serum dilution, this level is about 6-fold above our LoD, implying that this assay could potentially be used for detecting clinically relevant concentrations of PA during early infection.

We also explored the thermal and long-term storage capabilities of the PCC agent and nanostructured ELISA electrode platform. This is relevant to many potential field uses, since all components of the assay would need to be available immediately upon demand. A lyophilized sample of biligand was stored for 5 days at 65 °C under nitrogen atmosphere. Analytical HPLC traces (Figure 4a) and MALDI-TOF (Figure 4b) reveal that the chemical fingerprint of the capture agent remained unchanged during this period. The capture agent elutes as an isobaric doublet on analytical HPLC before and after thermal treatment (see Figure S10 for full time course). This doublet is likely due to spontaneous isomerization, a commonly encountered effect in peptide synthesis that can be accelerated at elevated temperatures.[37] The colorimetric ELISA assay performance of the PCC Agent remained unaffected after thermal treatment. The assembled electrochemical ELISA electrode also showed no drop in performance after storage in buffer at 40 °C for one week. We also tested the electrochemical ELISA at 0, 37, and 50 °C (in addition to the room temperature tests) and the assay performed well at all temperatures. A slight diminishment in output current was observed at 50 °C, which likely represents degradation of the PA antibody or the enzyme label. This modest drop in performance at highly elevated temperature could be addressed by the use of a nonenzymatic amplification strategy, such as electroactive nanoparticles or liposomes loaded with electrochemically active molecules.[38]

CONCLUSIONS

To implement a detection assay in the field, the assay must be robust to demanding physical conditions, and it must exhibit a high level of sensitivity and selectivity for detecting the target from samples such as groundwater or serum. Using the detection of *B. anthracis* exotoxin PA as an example, we report on an approach for designing such an immunoassay that can meet these requirements. Our approach is twofold. We first combined the technologies of bacterial display libraries and *in situ* click screening to develop a PCC Agent biligand that serves as a thermally stable anti-PA capture agent. That biligand is designed to serve as a drop-in replacement for the capture antibody in a sandwich-type ELISA assay. We then combined nanostructured gold electrodes, a DNA-based surface chemistry, and the anti-PA capture agent to construct an electrochemical ELISA assay for the detection of PA. When tested in 1% serum spiked with PA, the assay exhibits a limit of detection of <3 pM. The basic approach described here should be applicable toward developing biodetection assays against other important targets.

EXPERIMENTAL SECTION

Dimethyl sulfoxide (DMSO), dimethyl formamide, *N*-methyl pyrrilidone (NMP), mercaptohexanol (MCH), streptavidin, and gold(III) chloride hydrate 99.999% were purchased from Sigma. 4-Aminophenylphosphate monosodium salt hydrate (APP) was obtained from Gold Biotechnology (USA). Skim milk protein was purchased from Lab M limited (Lancashire, U.K.). All chemicals and solvents used were analytical grade of the highest purity available and were used as received. Purified *B. anthracis* Protective Antigen was generously provided by BEI Resources (NR-3780) and used in assays and as a screening target. Thiol-modified ssDNA and cDNA oligomers were synthesized and purified by Integrated DNA Technologies, Inc. (Coralville, IA). The concentrations were quantified by OD_{260} based on their individual absorption coefficients. Their sequences were

Thiol modified ssDNA: 5′-SH-AAAAAAAAAAAAAGAGTAGCCTTCCCGAGCATT-3′

cDNA: 5′-NH_3-AAAAAAAAAAA AAATGCTCGGGAAGGCTACTC-3′

All buffer solutions were prepared using Milli-Q (18 MΩ) water (Millipore) as a solvent. The buffer solutions employed in this work were as follows. DNA immobilization buffer: phosphate-buffered saline (PBS 1×) from Invitrogen (Carlsbad, CA). Reaction buffer for APP: 50 mM Tris−HCl (pH 9.6) containing 10 mM $MgCl_2$. Buffer for other chemicals: Tris-buffered saline (TBS 1×). Rinsing for removal of all nonspecific binding on the surface: 1× TBS with 0.1% Tween.

Multiple affinity, selectivity, biochemical, and biological assays are described in this work. Full experimental procedures, synthetic protocols, and additional experimental data are discussed in the Supporting Experimental Methods.

Preparation of Anchor. The anchor peptide was isolated from the eCPX bacterial display library (Cytomx Therapeutics; San Francisco, CA) using a microfluidic magnetic sorter (MMS; Cynvenio Biosystems) as described previously.[30] The bacterial display anchor peptide was synthesized with a C-terminal biotin label, and an N-terminal propargyl glycine to form Anchor-1 (Figure S1).

Biligand Screen. The *in situ* click screen starts with (a randomly selected) 65% of a ~1.8 million-element OBOC library of 5-mer peptides that is comprehensive in the D-stereoisomers of the

20 natural amino acids, excepting cysteine and methionine. The library was synthesized on TentaGel (Rapp Polymere) using standard Fmoc Solid Phase Peptide Synthesis protocols on an Aapptec Titan 357 automated peptide synthesizer (Supporting Experimental Methods) using split and mix protocols. The screen, which was carried out in blocking buffer (25 mM Tris-Cl (pH = 7.5), 150 mM NaCl, 0.1% (v/v) Tween-20, and 5% dehydrated milk), proceeds through several stages (Figure 1a) and begins with 500 mg of side-chain deprotected and blocked library beads. For each stage, potential hit beads are identified through the use of alkaline phosphatase (ALP)-labeled antibodies (see Supporting Experimental Methods for details). The first stage is designed to detect and remove those beads (typically <10% of the library) that bind to any (non-PA) reagents that are employed in the screen. Stage 2 screens for those beads that exhibit binding to PA. For this stage, 7 mM of Anchor-1 + 700 nM PA are incubated in 20 mL of blocking buffer with the OBOC library. Target binding was detected using monoclonal anti-Protective Antigen (US Biotech) and an anti-mouse ALP conjugated antibody (Abcam). Those hits are then incubated in 1% human serum in blocking conditions for 60 min. Beads exhibiting off-target binding are detected using rabbit polyclonal pan anti-human serum (Abcam). The last stage, which is unique to the *in situ* click screen, is to analyze each of the hit beads for the presence of clicked product.[18] This screen uses streptavidin-ALP (Invitrogen) to detect the biotin presented by Anchor-1 as a means of detecting the *in situ* clicked product. This screen yielded 8 hits, which were sequenced by Edman degradation on an Applied Biosystems 494 cLC protein sequencer (Table S1). They exhibited a high homology, which is a signature of a successful *in situ* click screen. All eight candidate PCC Agent biligands were synthesized for testing using Cu-catalyzed click and standard SPPS protocols (Supplementary Methods).

Preparation of Electrochemical Sensor. For the electrochemical ELISAs, 1.5 mm diameter nanostructured gold electrodes were fabricated using a soft-lithography process. The positive GXR-601 photoresist was patterned on a glass substrate sputtered with 300 nm gold and 30 nm chromium for adhesion. After the lithography patterning of the electrodes, the substrate was immersed in gold etchant (Sigma Aldrich) for removal of the gold and chromium. A PDMS microwell was used to contain the buffer samples in contact with the electrodes, and was formed using a 5 mm PDMS punch. The bonding between the silica substrate and the PDMS was achieved *via* an O_2-plasma process. Those electrodes were cleaned in acetone with sonication (15 min), methanol (10 min), and DI water (10 min), and then dried under flowing N_2. They were immersed in 3 mg/mL gold(III) chloride hydrate solution in 0.5 M H_2SO_4 for electroplating of the nanostructured gold surface. Under constant stirring conditions, a potential of −400 mV (*vs* Ag/AgCl) was applied. The deposition parameters were optimized by measuring the active surface area by cyclic voltammetry and AFM (Park Systems XE-100 using a PPP-NCHR 10 M noncontact cantilever tip) upon varying deposition time and voltages (Figure S4).[32,33] All AFM images were obtained at 45 μm × 45 μm scan size and 0.5 Hz scan rate. Assuming a specific charge of 388 μC/cm^2 is required for gold oxide reduction,[34] the optimal nanostructured gold electrode had a total active surface area of 122.47 ± 1.5 mm^2 after 400 s of deposition, while the unmodified electrode had a total active surface area of 20.4 ± 0.8 mm^2. After deposition, the nanostructured gold electrodes were rinsed thoroughly, cleaned under flowing N_2, and stored in a desiccator.

DNA Monolayer Formation. A 200 μM solution of DNA in DMSO and deionized water (v/v = 1:1, total volume 10 μL) was drop-cast on the gold electrode and left overnight in a desiccator to allow the solvent to evaporate completely. The DNA-modified electrode was further treated with 30 μL of drop-cast 1.0 mM mercaptohexanol for 2 h,[39,40] followed by washing with water The fluorescence microscopy approach of Lee *et al.*[36] was employed to find optimized conditions for the DNA surface modification. For the hybridization, the electrode was immersed in 500 nM cDNA conjugated with streptavidin in 10 μL of TBS for 1 h at room temperature, followed by immersion in a 500 nM solution of the biotin-conjugated PCC Agent in 10 μL of 0.5% dehydrated milk in TBS for 1 h at 4 °C. After PCC Agent immobilization, the electrodes were blocked with 30 μL of blocking buffer (5% dehydrated milk in TBS) at 4 °C overnight.

Electrochemical ELISA. A preblocked electrode was incubated with nine concentrations ranging from 1 pM to 10 μM of the target PA protein, spiked into solutions of 0%, 1%, and 5% human serum, diluted in 0.5% milk in TBS for 1 h at 4 °C The electrode was then incubated in a solution containing alkaline phosphatase conjugated detection antibodies (anti-Protective Antigen; US Biotech) at 100 nM prepared in 0.5% milk in TBS. The electrode was then extensively rinsed with washing buffer (1× TBS + 0.05% Tween).

Cyclic voltammetry (CV) was performed using an electrochemical analyzer (CHI760D, CH Instruments) at room temperature unless otherwise indicated, using the gold electrode as the working electrode, an Ag/AgCl (sat. KCl) reference electrode and a Pt counter electrode. The potential was varied from −0.2 to 0.3 V with a scan rate of 20 mV s^{-1}. The solution was a 50 mM Tris−HCl (pH 9.6) with 10 mM $MgCl_2$ containing 20 mM APP. Electrochemical signals consisting of the oxidation of the APP substrate by the enzyme-linked antibodies on the electrode surface were monitored. Other sensitive electrochemical techniques, including differential pulse voltammetry (DPV) and chronoamperometry, could be used to monitor the oxidation current of the APP species. This requires no change in setup, but can provide a means of reducing noise that arises from stray capacitances. It was not used here because measured background current was found to be consistently very low and reproducible using CV. DPV might be a more appropriate measurement technique for field tests, where noise sources would be expected to be higher.

Conflict of Interest: The authors declare the following competing financial interest(s): James R Heath is a founder and board member of Integrated Diagnostics, which is seeking to commercialize the PCC Agent technology. Heather Agnew and Bert Lai are employees of Integrated Diagnostics. The patent "Multi-ligand capture agents and related compositions, methods and systems" (WO2009155420 A1) by H Agnew *et al.* was published December 23, 2009.

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Supporting Information Available: Figures and protocols outlining the details of the screening strategy and screening hits, affinity and specificity assays, as well as the various steps of optimization of the electrochemical electrode fabrication and surface modification. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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